Antibiofilm Activities of Ultrashort Antimicrobial Lipopeptides and Self-assembled Ultrashort Peptide Gels

Laverty, G., & Gilmore, B. (2013). *Antibiofilm Activities of Ultrashort Antimicrobial Lipopeptides and Self-assembled Ultrashort Peptide Gels*. Paper presented at 3rd International Meeting on Antimicrobial Peptides, London, United Kingdom. http://peptideconferences.org/imap/imap-2013

Antibiofilm Activities of Ultrashort Antimicrobial Lipopeptides and Self-assembled Ultrashort Peptide Gels

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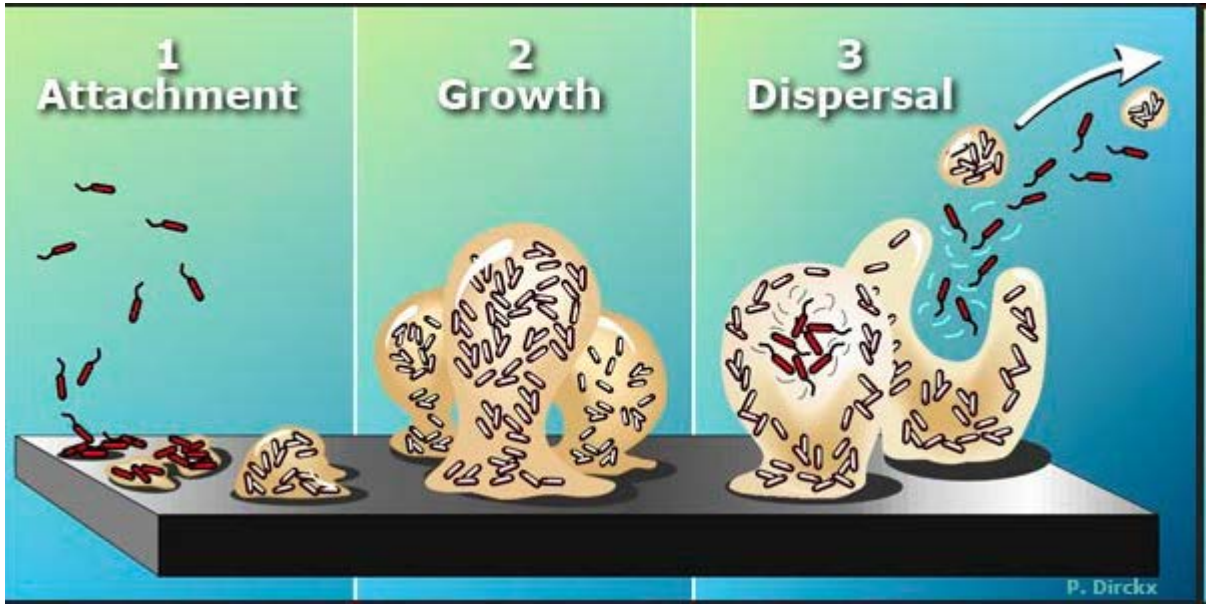
Queen's University Belfast

Planktonic vs. Biofilm Bacteria

- Planktonic form: Free floating in liquid
- Biofilm form: sessile, composed of aggregated microcolonies of cells surrounded by a protective extracellular polymeric matrix
- Mature biofilms can resist 10-1000 times the concentrations of standard antibiotic regimens that are required to kill genetically equivalent planktonic forms



P. Dirckx, Centre for Biofilm Engineering, Montana State University, Bozeman

Characteristics and Treatment of Medical Device Associated Infections. Laverty, G. and Gilmore, B.F. Book title: Advances in Medicine and Biology. Volume 51. Eds. Berhardt, L.V. Nova Science Publishers Inc

Calgary Biofilm Device (MBEC plate)

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D. & Buret, A. 1999, "The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms", *Journal of clinical microbiology,* vol. 37, no. 6, pp. 1771-1776.

Antimicrobial Peptides in Nature: Amphibian *Bombina maxima*

- Maximin-4

GIGGVLLSAGKAALKGLAKVLAEKYAN-NH_2

- Primary sequence: 27 amino acids
- Lowest Minimum Inhibitory Concentration (MIC) value of 2.7 μg/mL against *Staphylococcus aureus*

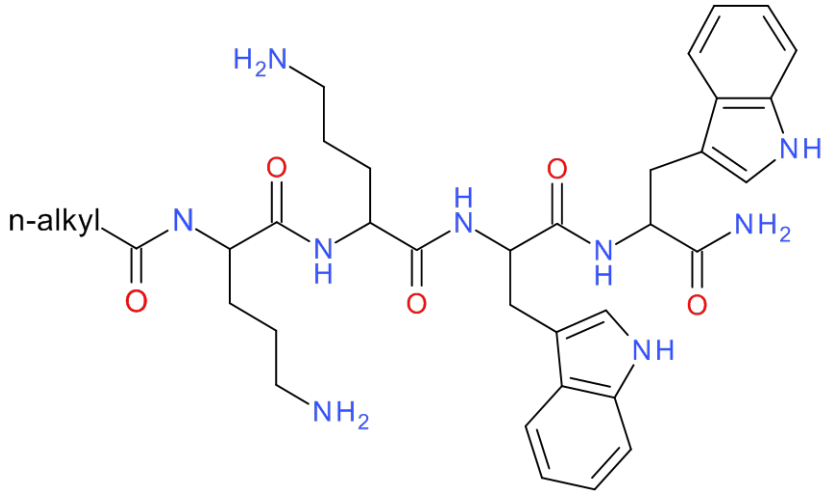
Lai, R., Zheng, Y.T., Shen, J.H., Liu, G.J., Liu, H., Lee, W.H., Tang, S.Z. & Zhang, Y. 2002, "Antimicrobial peptides from skin secretions of Chinese red belly toad *Bombina maxima*", *Peptides,* vol. 23, no. 3, pp. 427-435.

Rational Design and Selection of an Antimicrobial Peptide Motif

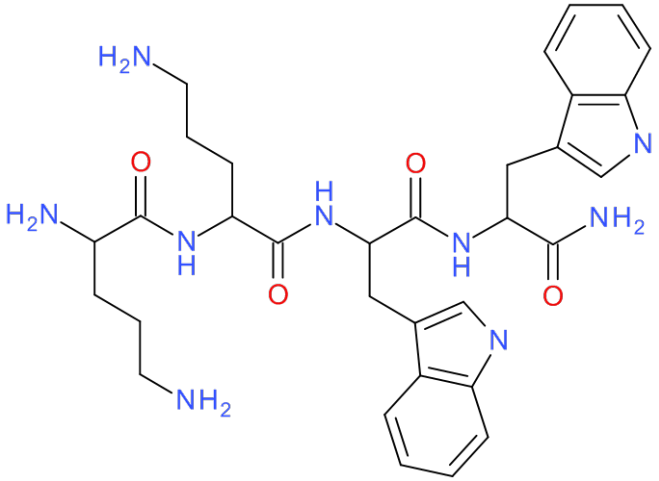
- Structure, more specifically the **hydrophobic: charge ratio** is more important with regard to antimicrobial activity than size
- Manipulation of primary amino acid sequence can improve factors such as:
  - Specificity
  - Toxicity
  - Stability
- Ultra short antimicrobial peptides consist of approximately four or five amino acids residues,
- Amino acid selection fulfils the minimum range and balance of functionalities:
  - Charge
  - Lipophilicity

Laverty, G., Gorman, S.P. and Gilmore, B.F (2011). Antimicrobial Peptides as Biocides. *International Journal of Molecular Sciences* **12 (10)**; 6566-6596.

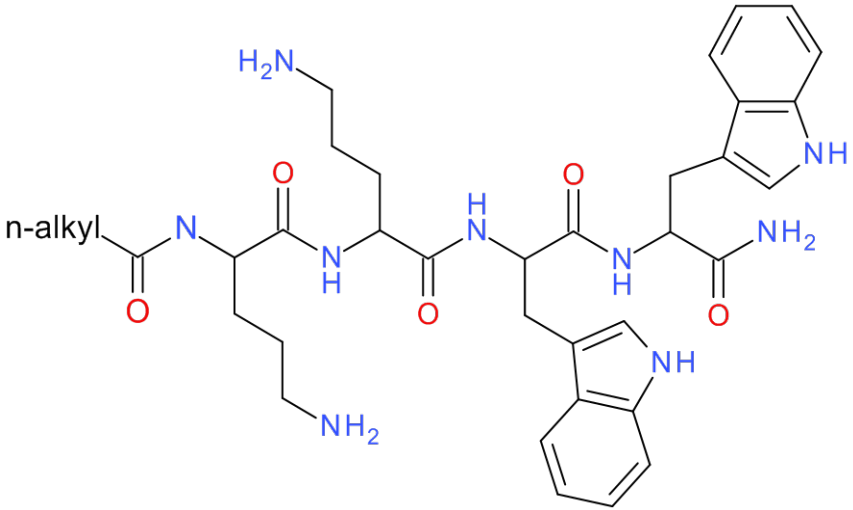
Ultrashort Antimicrobial Lipopeptides



Cationic Ultra short Antimicrobial Peptides



Structure of H_2N-OOWW-NH_2, RMM: 617.34
* O = Ornithine

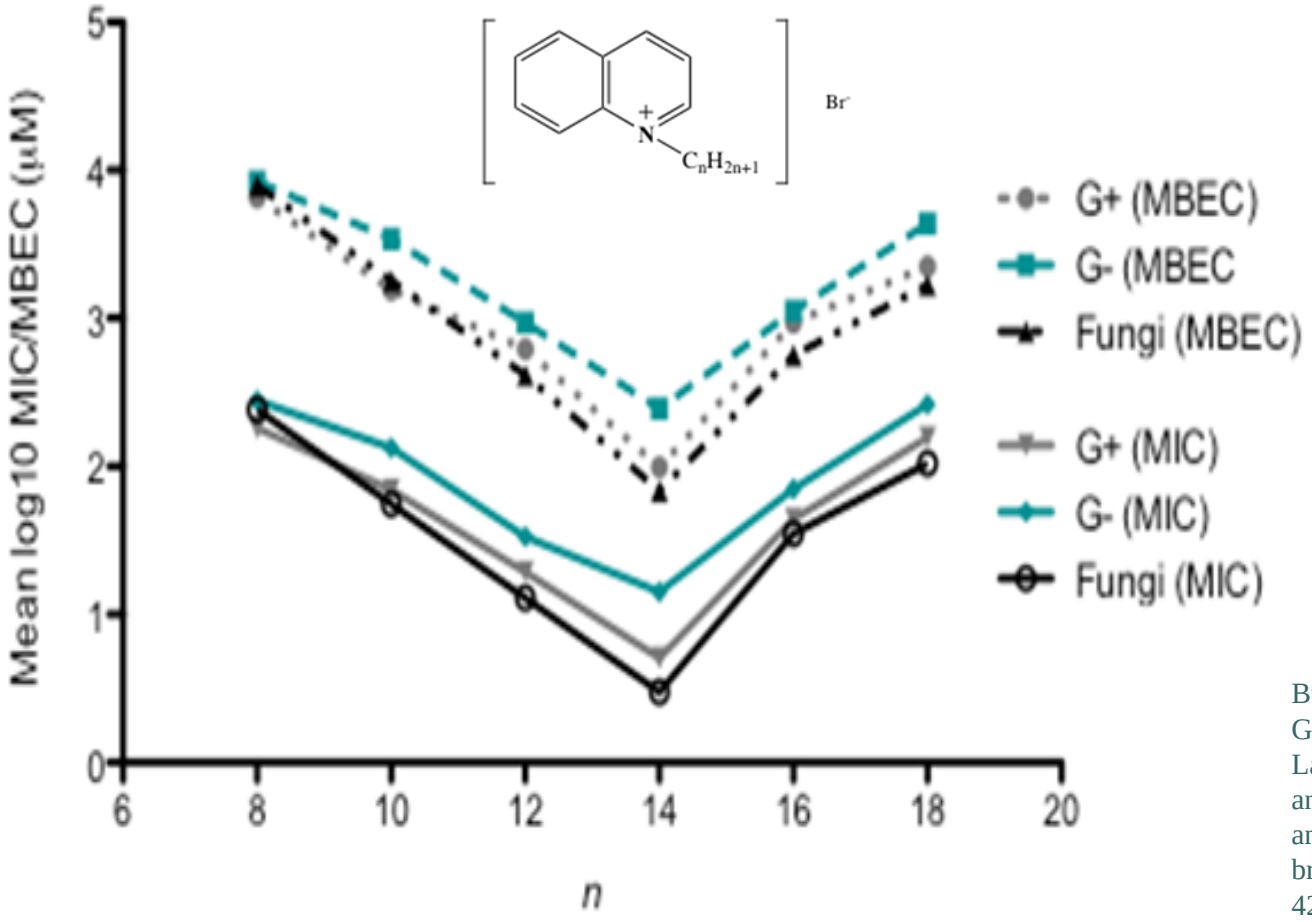


Structure of n-alkyl-OOWW-NH2

***n-alkyl represents the addition of a hydrocarbon based acid moiety**

Bisht, G.S., Rawat, D.S., Kumar, A., Kumar, R. & Pasha, S. 2007, "Antimicrobial activity of rationally designed amino terminal modified peptides", *Bioorganic & medicinal chemistry letters,* vol. 17, no. 15, pp. 4343-4346.

Lipophilic: Charge Balance. Addition of Fatty acids: 1-alkylquinolinium bromide Ionic Liquids



G+ve microorganisms:

- MRSA (ATCC 43300)
- *S. epidermidis* (ATCC 35984)
- *S. aureus* (ATCC 29213)

G-ve microorganisms:

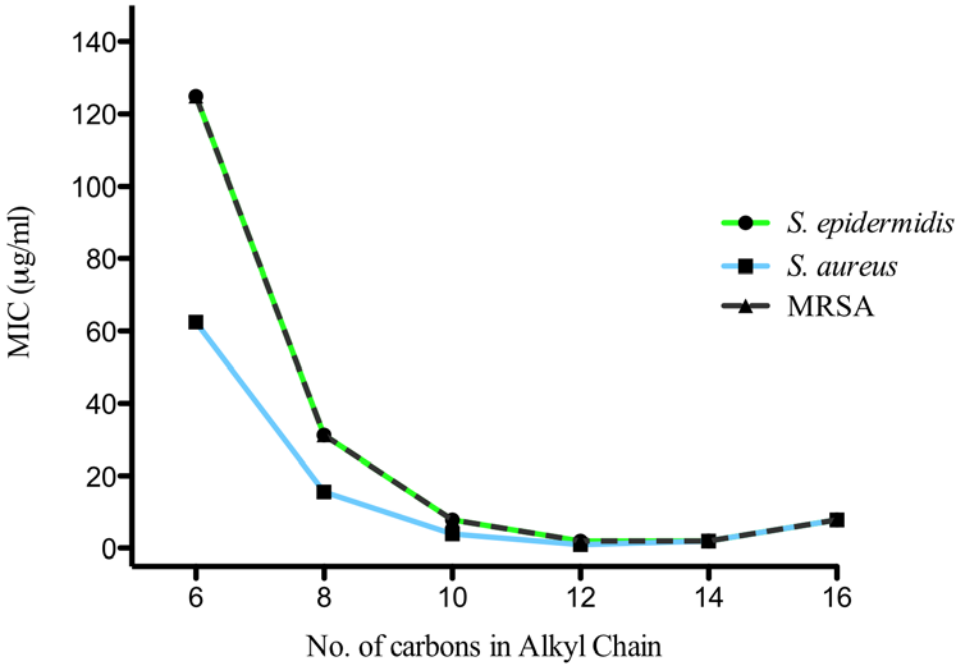
- *E. coli* (NCTC 8196)
- *K. aerogenes* (NCTC 7427)
- *B. cereus* (NCTC 2599)
- *P. mirabilis* (NCTC 12442)
- *P. aeruginosa* (PAO1)

Fungus:

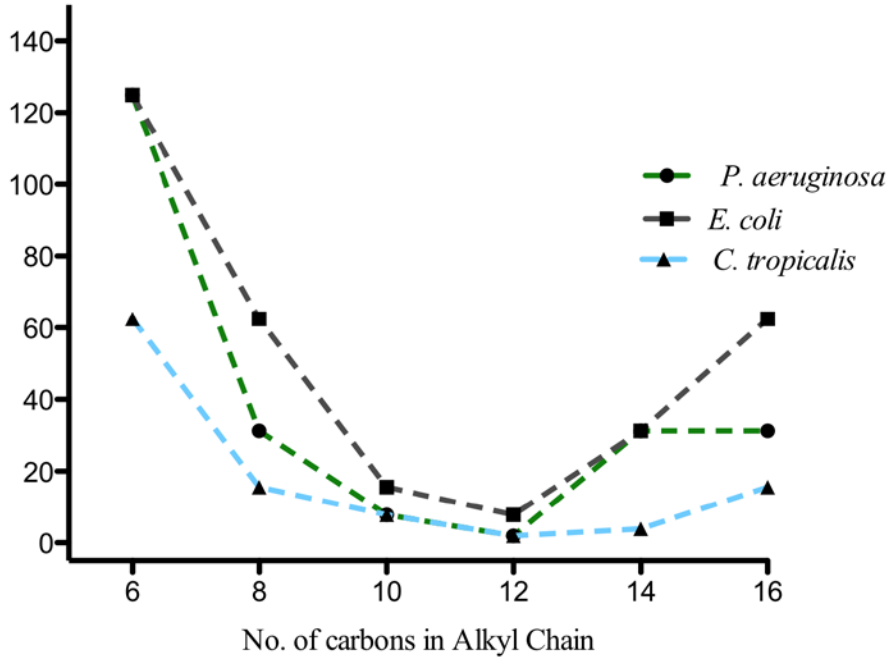
- *C. tropicalis* (NCTC 7393)

Busetti, A., Crawford, D.E., Earle, M.J., Gilea, M.A., Gilmore, B.F., Gorman, S.P., Laverty, G., Lowry, A.F., McLaughlin, M. and Seddon, K.R. (2010) Antimicrobial and antibiofilm activities of 1-alkylquinolinium bromide ionic liquids. *Green Chemistry* **12**; 420 - 425.

Lipophilic: Charge Balance
Addition of Fatty acids to tetrapeptide



Vs. Gram-positive bacteria



Vs. Gram-negative bacteria and a fungus

Laverty, G., McLaughlin, M., Shaw, C., Gorman, S.P. and Gilmore, B.F. (2010) Antimicrobial Activity of Short, Synthetic Cationic Lipopeptides. *Chemical Biology and Drug Design* **75 (6)**; 563-569

Anti-Biofilm Results

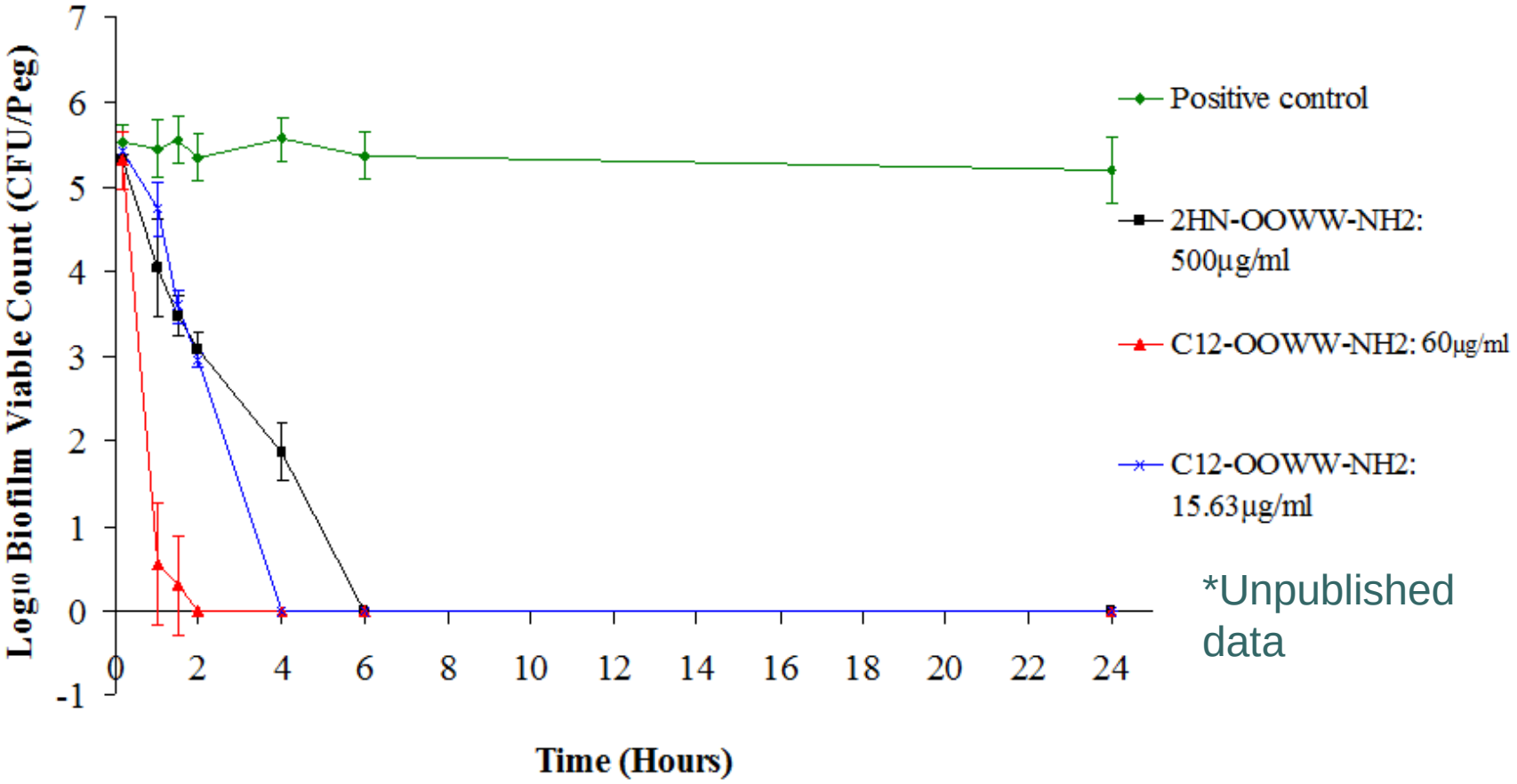
Concentrations all (µg/mL)

		Fmoc	NH2	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}
***S. epidermidis* (MRSE)**	MIC	15.63	125	125	31.25	7.81	1.95	1.95	7.81
ATCC 35984	MBEC	63.5	500	>1000	>1000	250	15.63	15.63	15.63
***S. aureus* ATCC 29213**	MIC	3.91	125	62.5	15.63	3.91	0.95	1.95	7.81
	MBEC	500	500	>1000	>1000	500	62.5	31.25	62.5
***S. aureus* ATCC 43300**	MIC	7.81	250	125	31.25	7.81	1.95	1.95	3.91
(MRSA)	MBEC	250	500	1000	1000	1000	62.5	62.5	62.5
***P. aeruginosa* PA01**	MIC	125	250	125	31.25	7.81	1.95	31.25	31.25
	MBEC	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
***E. coli* NCTC 8196**	MIC	62.5	500	125	62.5	15.63	7.81	31.25	62.5
	MBEC	>1000	>1000	>1000	>1000	>1000	500	>1000	>1000
***C. tropicalis* NCTC 7393**	MIC	15.6	125	62.5	15.63	7.81	1.95	3.91	15.63
	MBEC	>1000	>1000	>1000	>1000	250	250	>1000	>1000

Current Antibiotics

Antimicrobial	*S. epidermidis* (ATCC 35984)			*S. aureus* (ATCC 29213)			MRSA (ATCC 43300)		
	MIC (mg/L)	MBC (mg/L)	MBEC (mg/L)	MIC (mg/L)	MBC (mg/L)	MBEC (mg/L)	MIC (mg/L)	MBC (mg/L)	MBEC (mg/L)
Vancomycin	1.95	3.91	>1000	1.95	7.81	>1000	1.95	7.81	>1000
Rifampicin	1.95	1.95	62.5	0.24	0.98	15.63	1.95	1.95	>1000
Gentamicin	31.25	62.5	>1000	0.49	1.95	15.63	0.49	7.81	>1000
Trimethoprim	>1000	>1000	>1000	1.95	7.81	>1000	62.5	250	>1000
Ciprofloxacin	0.98	0.98	>1000	1.95	31.25	500	7.81	125	>1000

Rate of Kill: Established 24 hour Biofilms *S.epidermidis*



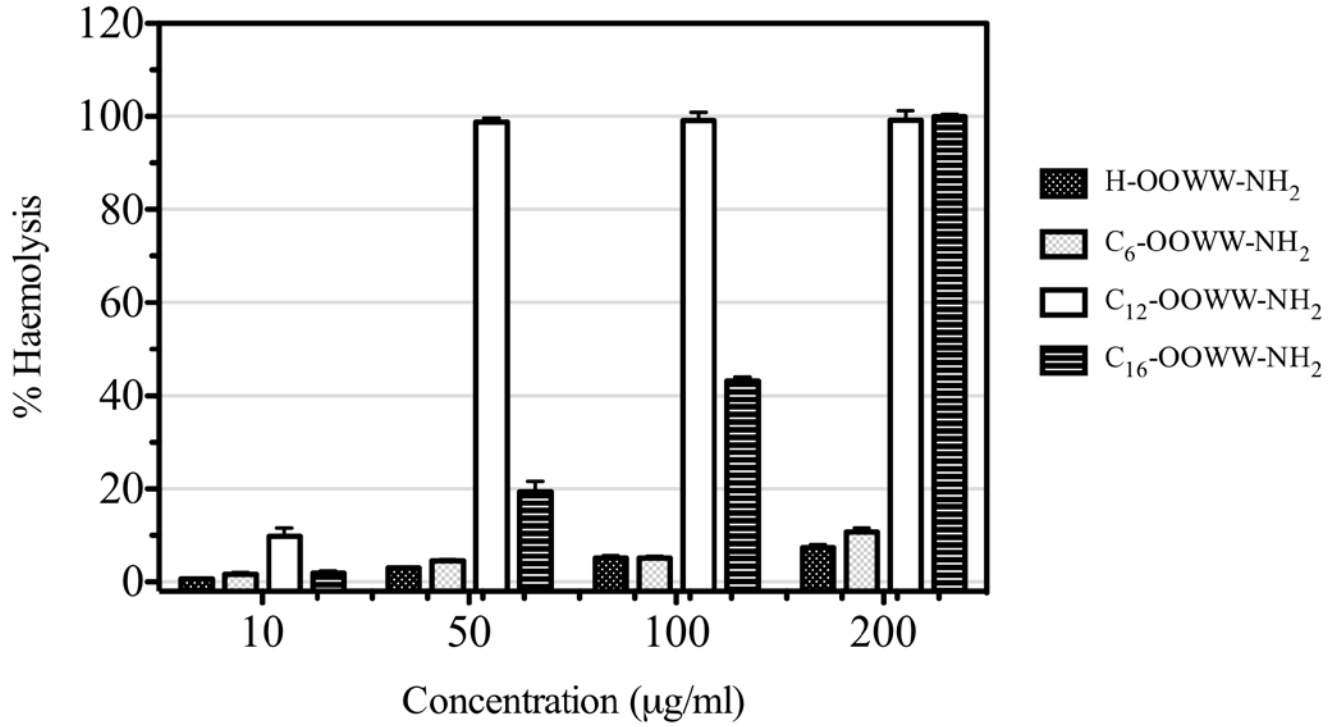
*Unpublished data

Rapid rate of kill: within 2 hours at 4x MBEC (60µg/mL)
within 4 hours at MBEC (15.63µg/mL)

Mechanism of action

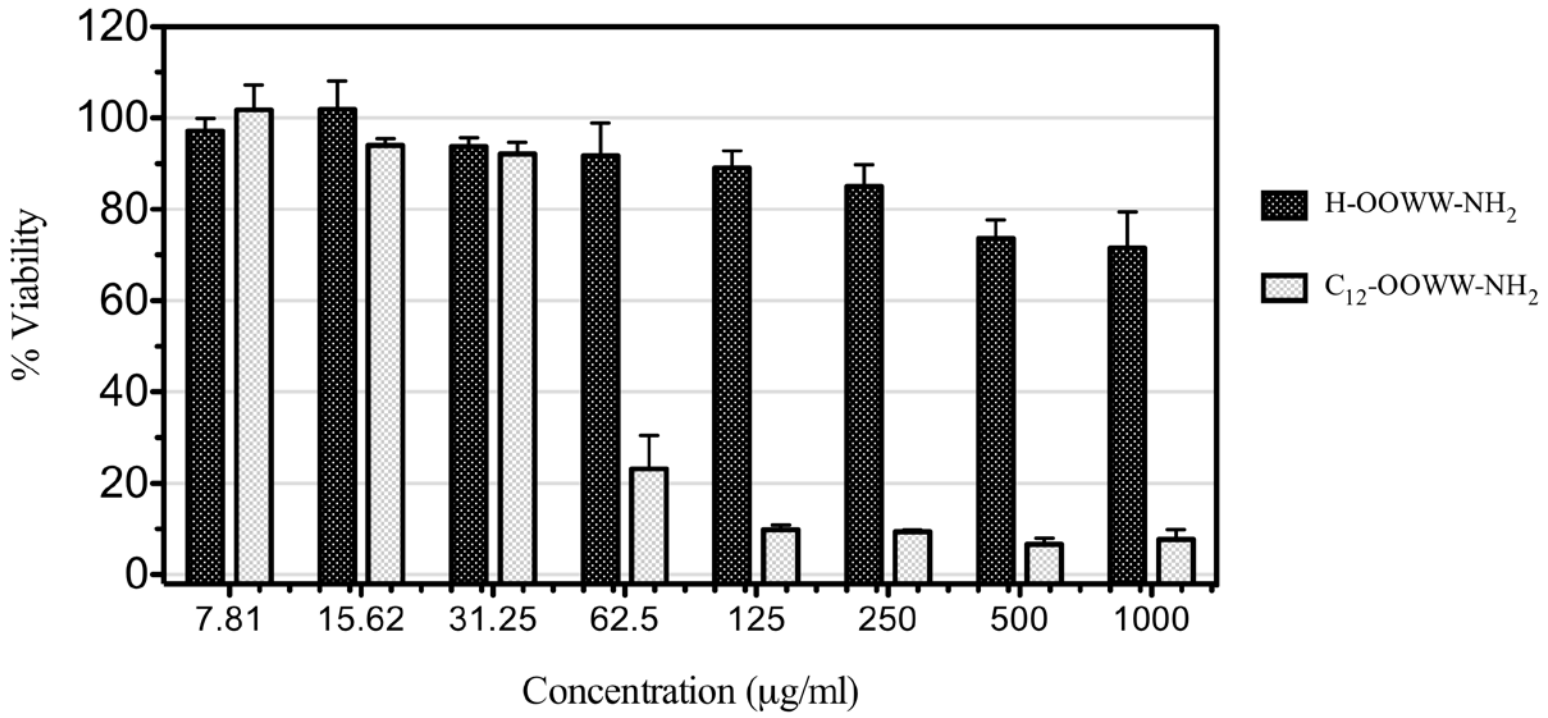
- Cationic antimicrobial peptide attracted to negatively charged bacterial membrane
- Inserts into membrane, forming pores, acts like a detergent, self promoting uptake of peptide
- Ultrashort Peptides known to be unordered in hydrophilic solution. Form α-helical structures in contact with hydrophobic environments e.g. cell membrane

Cytotoxicity: Haemolysis assay: 1 hour exposure



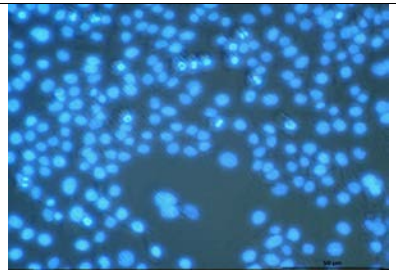
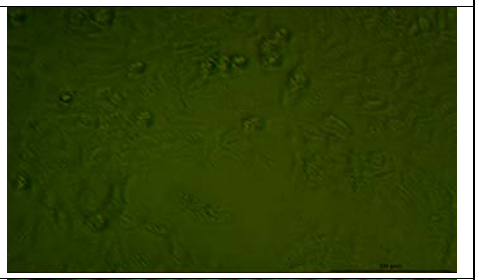
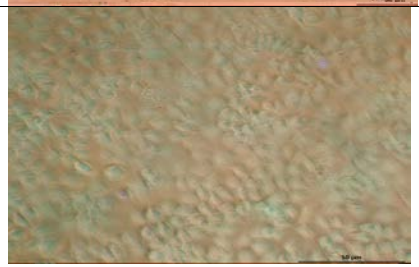
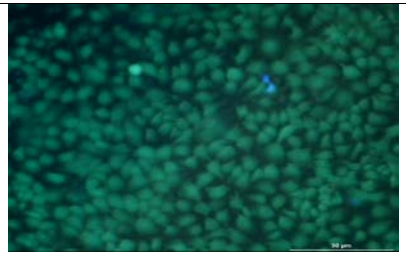
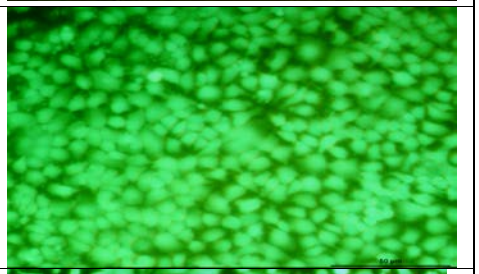
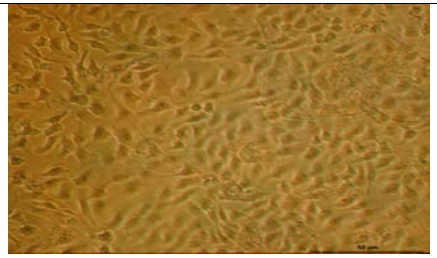
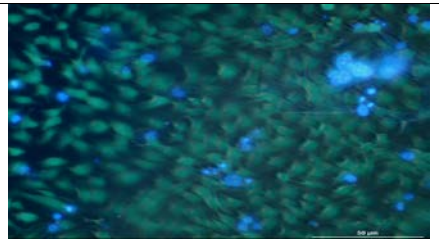
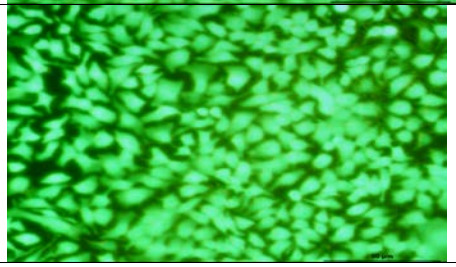
Haemolytic activity of the tetrapeptide amide and lipopeptides C*n*OOWW-NH_2 (where *n* = 6, 12 and 16) against equine erythrocytes. Each value is expressed as the mean of six replicates.

Cytotoxicity: Tissue Culture: MTT Assay: 24 hour exposure



Cytotoxicity of the tetrapeptide amine and the most potent antimicrobial lipopeptide, C_{12}OOWW-NH_2 evaluated against human keratinocyte (HaCaT) cells. Each value is expressed as the mean of six replicates.

Cytotoxicity: Fluorescence Microscopy: 1 hour exposure, 60x magnification

Concentration of C_{12}-OOWW-NH_2 (µg/ml)	Cell Image (555nm)	DAPI Excitation Wavelength (350nm) Presence of Blue = DAPI release from cells, cell membrane compromised	Calcein AM Excitation Wavelength (494nm). Presence of green = Calcein AM retained by cells, cells viable.
Total Kill (90% ethanol)			
Zero Kill			
15 µg/ml C_{12}-OOWW-NH_2			

Significance: Drug Delivery-Biomaterials

- Implants provide an inert surface for bacteria to adhere to and form biofilm
- Implant-associated infection increasingly prevalent problem:
  - Higher standards of living
  - Improvements in medicine
  - Increase in life expectancy and greater demand for medical devices to replace the normal physiological functioning of the aging human body
- Burden with respect to:
  - Healthcare budgets
  - Prolonged hospital stays
  - Patient mortality and morbidity

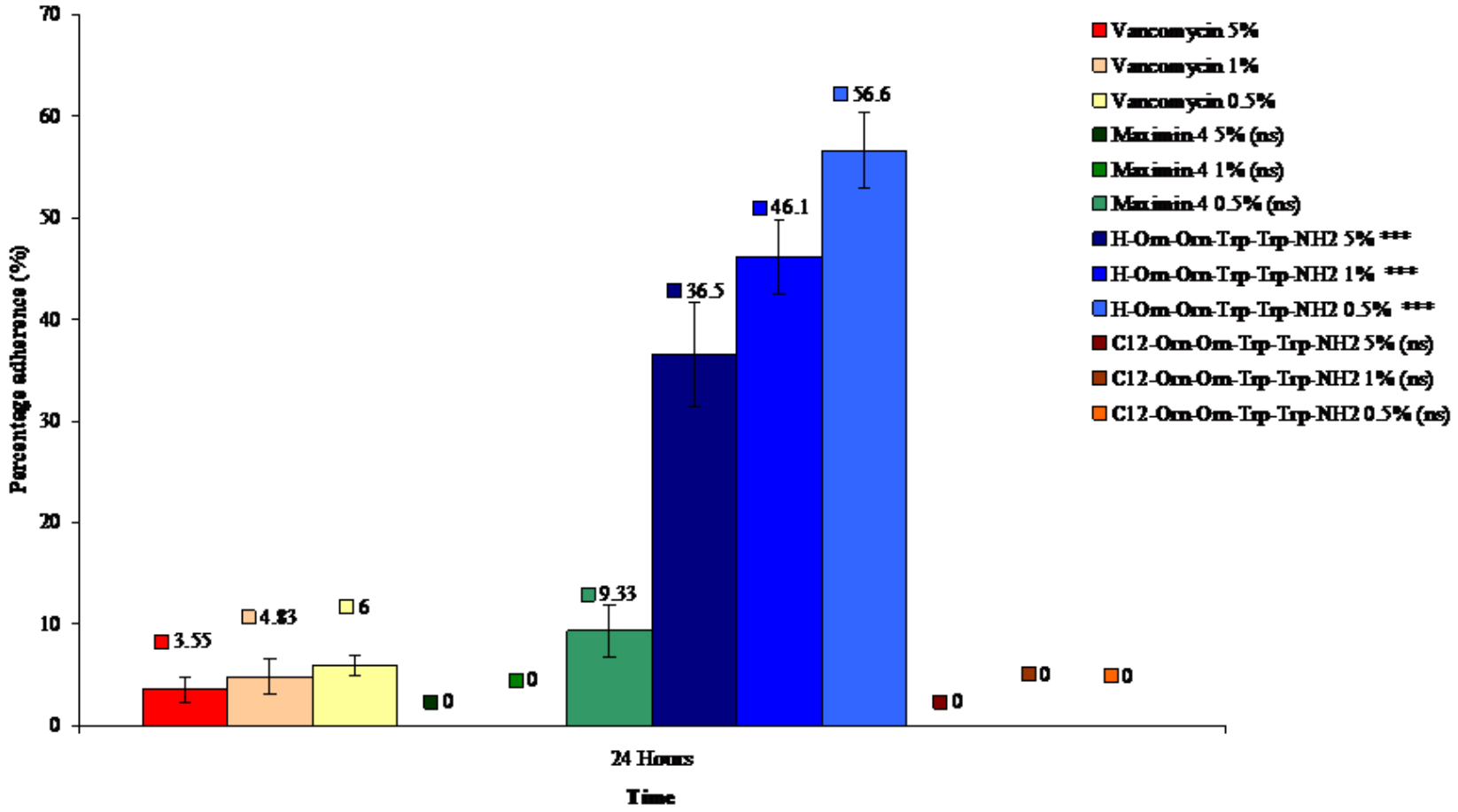
Biofilms and Implant-Associated Infections. Laverty, G., Gorman, S.P. and Gilmore, B.F. Book title: Biomaterials and Medical Device Associated Infections. Eds. Barnes, L. and Cooper, I. Woodhead Publishing Ltd. Due for release Winter 2013

Antimicrobial Hydrogel

- Hydrogels common coating utilised for medical devices such as catheters
- Absorbs water, a polymer with similar properties to tissue, biocompatible, flexible.
- Matrix and immersion loaded poly(2-hydroxyethyl methacrylate) (poly(HEMA)) hydrogels:
  - Maximin-4
  - H-Orn-Orn-Trp-Trp-NH_2
  - C_{12}-Orn-Orn-Trp-Trp-NH_2
  - Vancomycin
- Release of peptide/drug and adherence of *S.epidermidis* ATCC 35984 studied

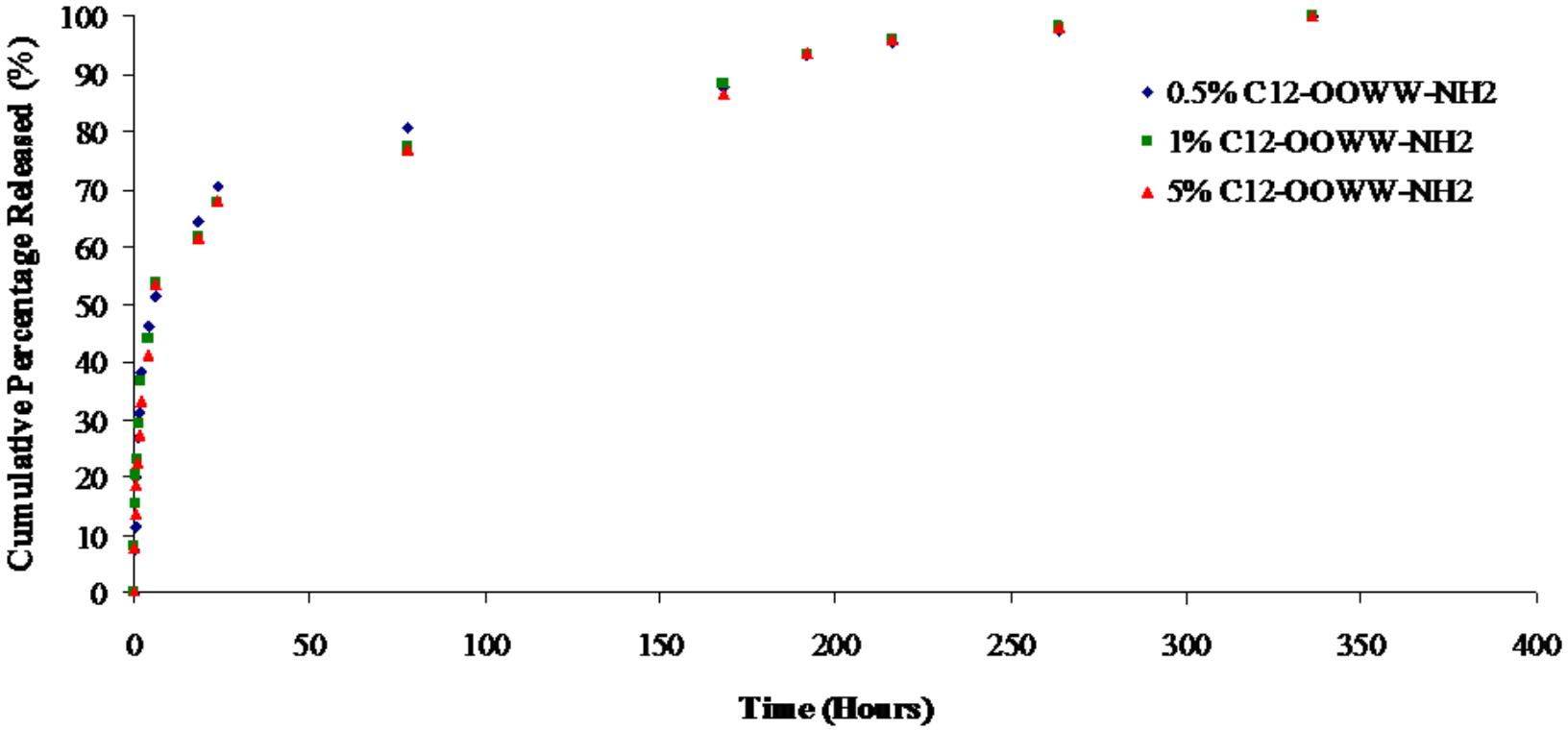
Laverty, G., Gorman, S.P. and Gilmore, B.F (2012). The Adherence of *Staphylococcus epidermidis* to Antimicrobial Peptide Incorporated poly(2-hydroxyethyl methacrylate) Hydrogels. *Journal of Biomedical Materials Research: Part A* **100A**; 1803–1814.

Adherence Results 24 hours



Mean % adherence of *S. epidermidis* ATCC 35984 to 0.5%, 1% and 5% vancomycin, maximin-4, H-Orn-Orn-Trp-Trp-NH_2 and C_{12}-Orn-Orn-Trp-Trp-NH_2 matrix loaded, 1% EGDMA crosslinked, poly(HEMA) hydrogels relative to positive control (no drug) after 24 hours. Results are displayed as the mean of five samples

Release Results: UV spectroscopy



The cumulative percentage drug release of C_{12}-Orn-Orn-Trp-Trp-NH_2 released (µg) from a 0.5%, 1% and 5% matrix loaded poly(HEMA) hydrogel into 37ºC 10mLs PBS, pH 7.4, over a period of 2 weeks. Results are displayed as the mean of five replicates. Concentrations obtained via UV-visible spectroscopy from a fresh standard calibration curve (five replicates) of equation y = 0.0075x (R2=0.999, 280nm)

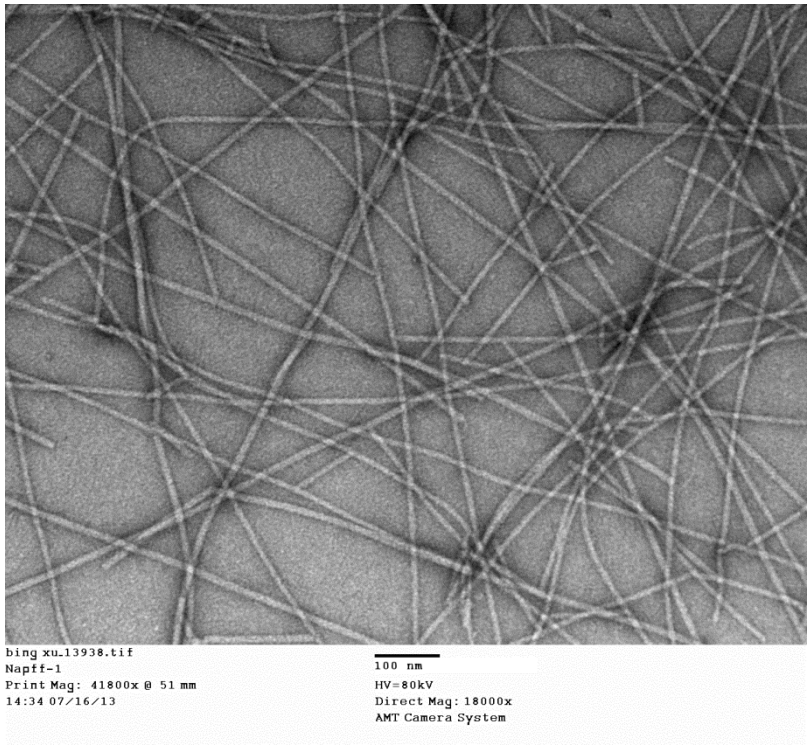
Self-assembled Ultrashort Peptide Gels

- 3 month Research Placement Prof. Bing Xu Lab, School of Chemistry, Brandeis, Waltham, Boston
- April - August 2013
- Successful in producing a series of ultrashort peptides that self-assembled at physiological pH
- Hydrophobicity provided by inclusion of a napthalene grouping and varying quantity of phenylalanine in primary structure
- Charge: Lysine and/or Ornithine

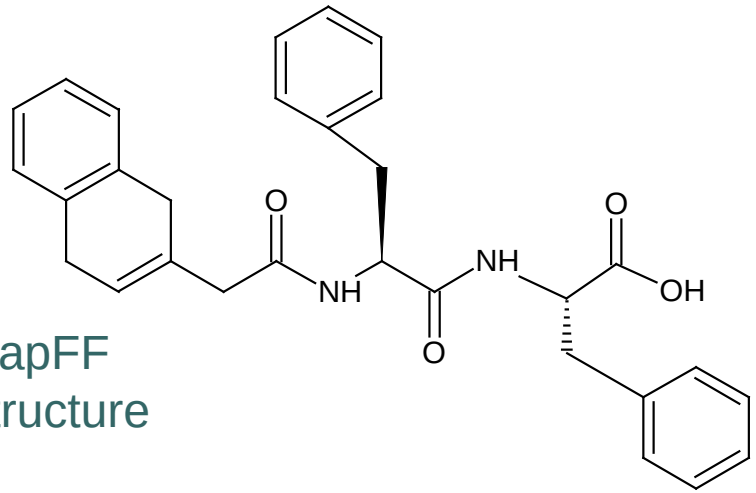
Queen's University Belfast

Preparation of Ultrashort Self-assembled Peptide Gels

- Nanofibre structures formed
- Due to π-π interactions between aromatic moieties
- B-sheets
- Cytocompatible: up to 200µM



TEM of 1%w/v NapFF showing nanofibre structures.



NapFF structure

Ultrashort Self-assembled Peptide Gels: NapFF

- Formed gels initially at concentrations (w/v):

Gelation concentration

2% NapFF

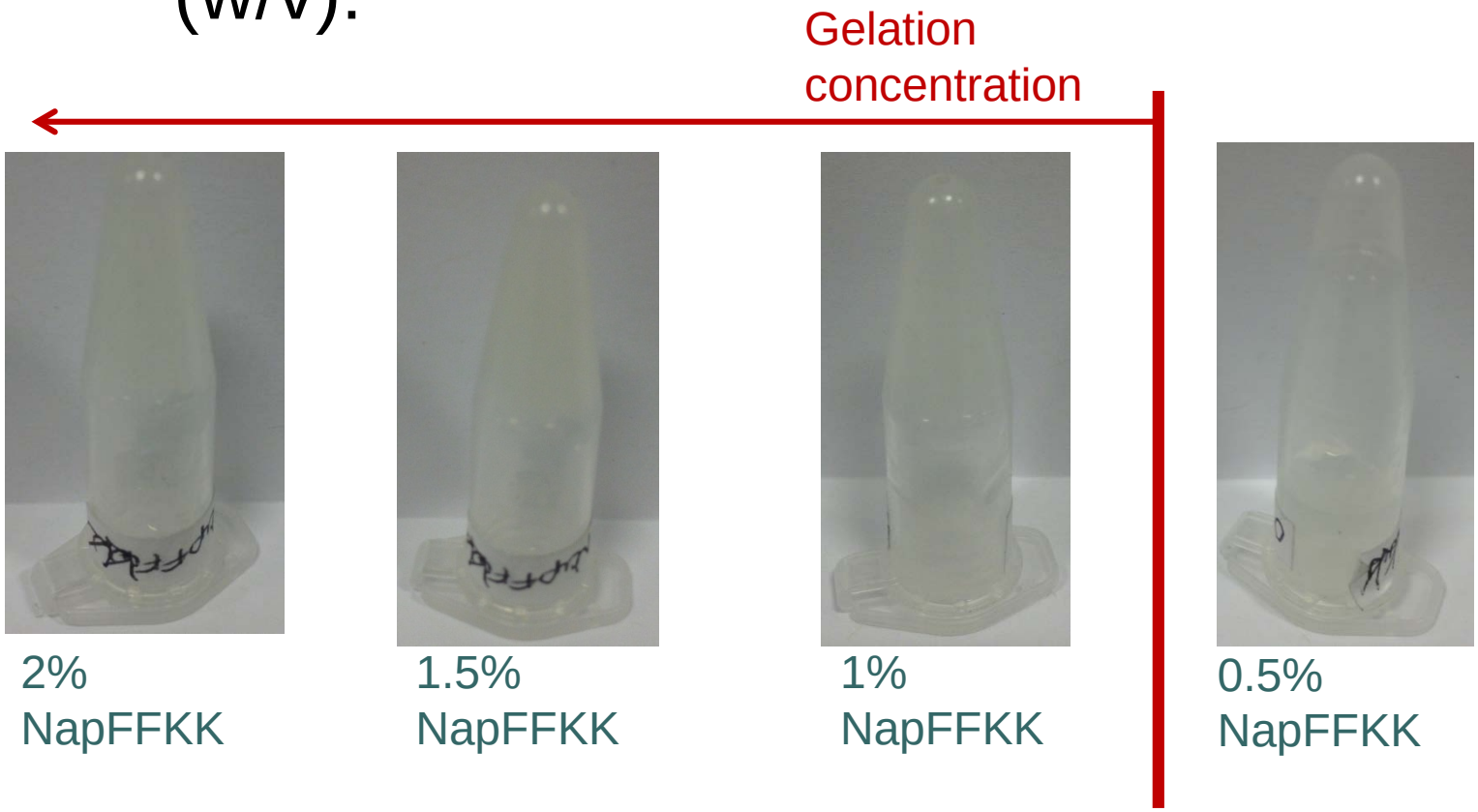
1.5% NapFF

1% NapFF

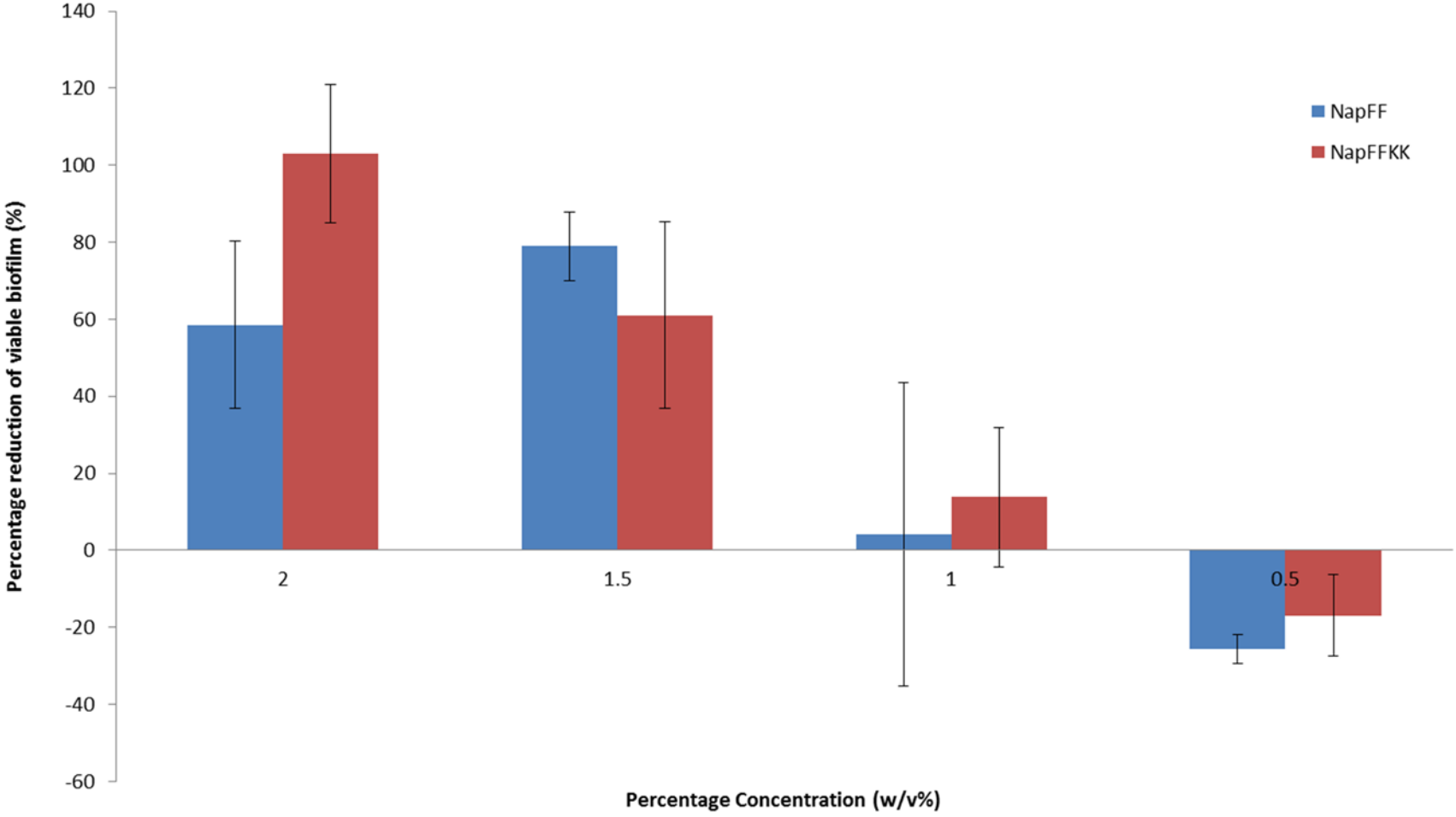
0.5% NapFF

Ultrashort Self-assembled Peptide Gels: NapFFKK

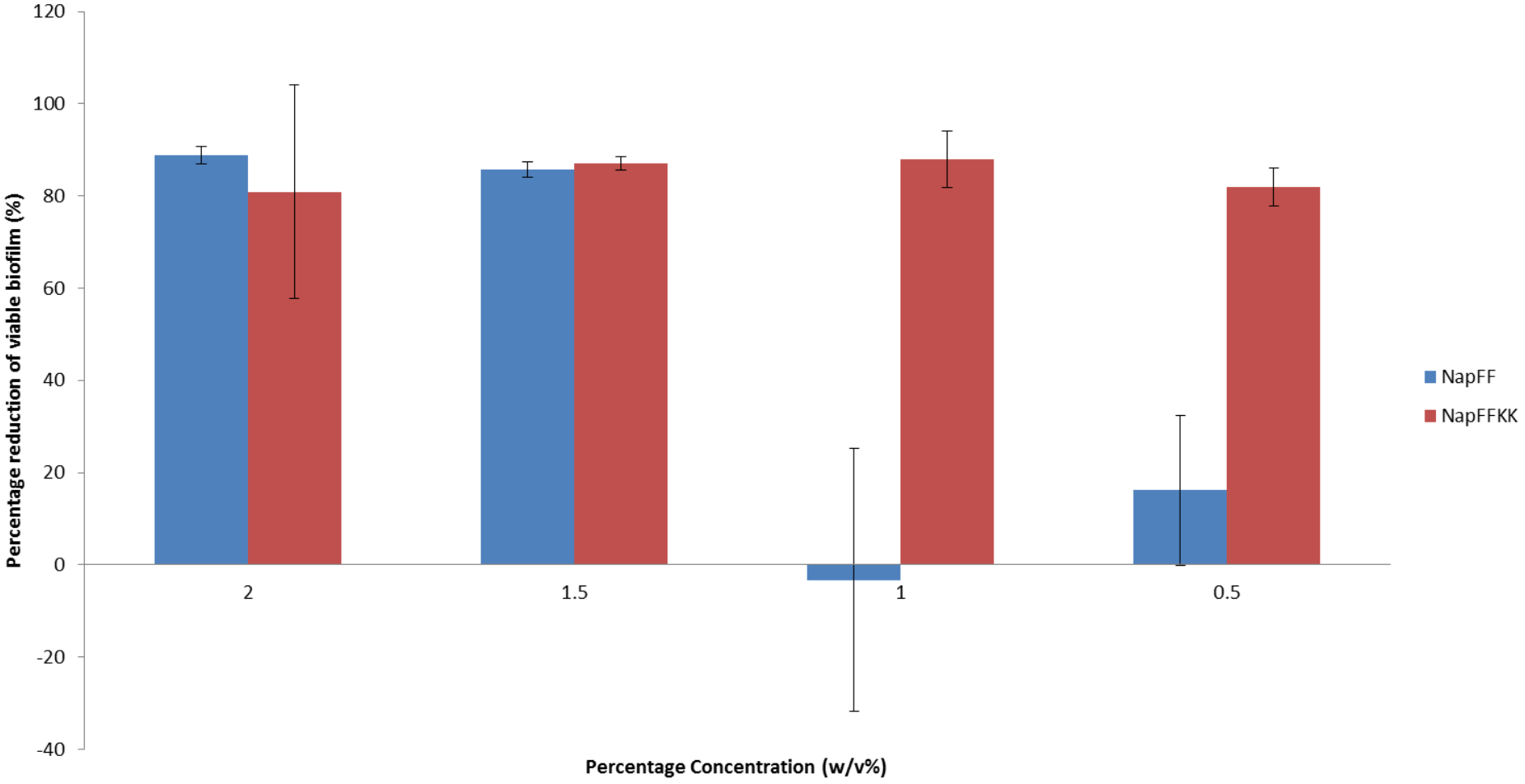
- Formed gels initially at concentrations (w/v):



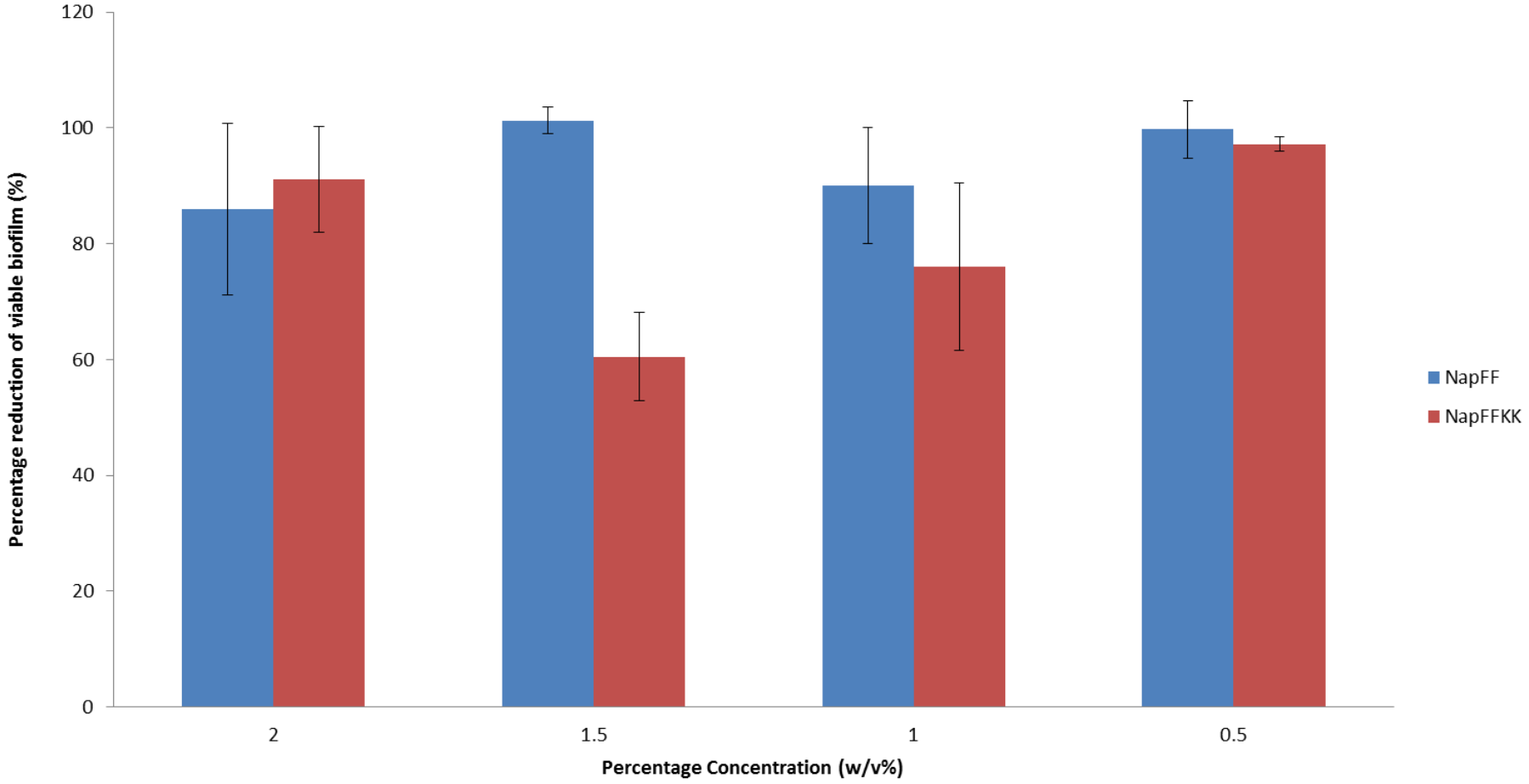
Percentage reduction of mature 24 hour *Staphylococcus epidermidis* ATCC 35984 biofilm after 24 hour incubation with NapFF and NapFFKK self-assembled hydrogels utilising an alamarBlue assay. Results are displayed as a mean of 8 replicates.



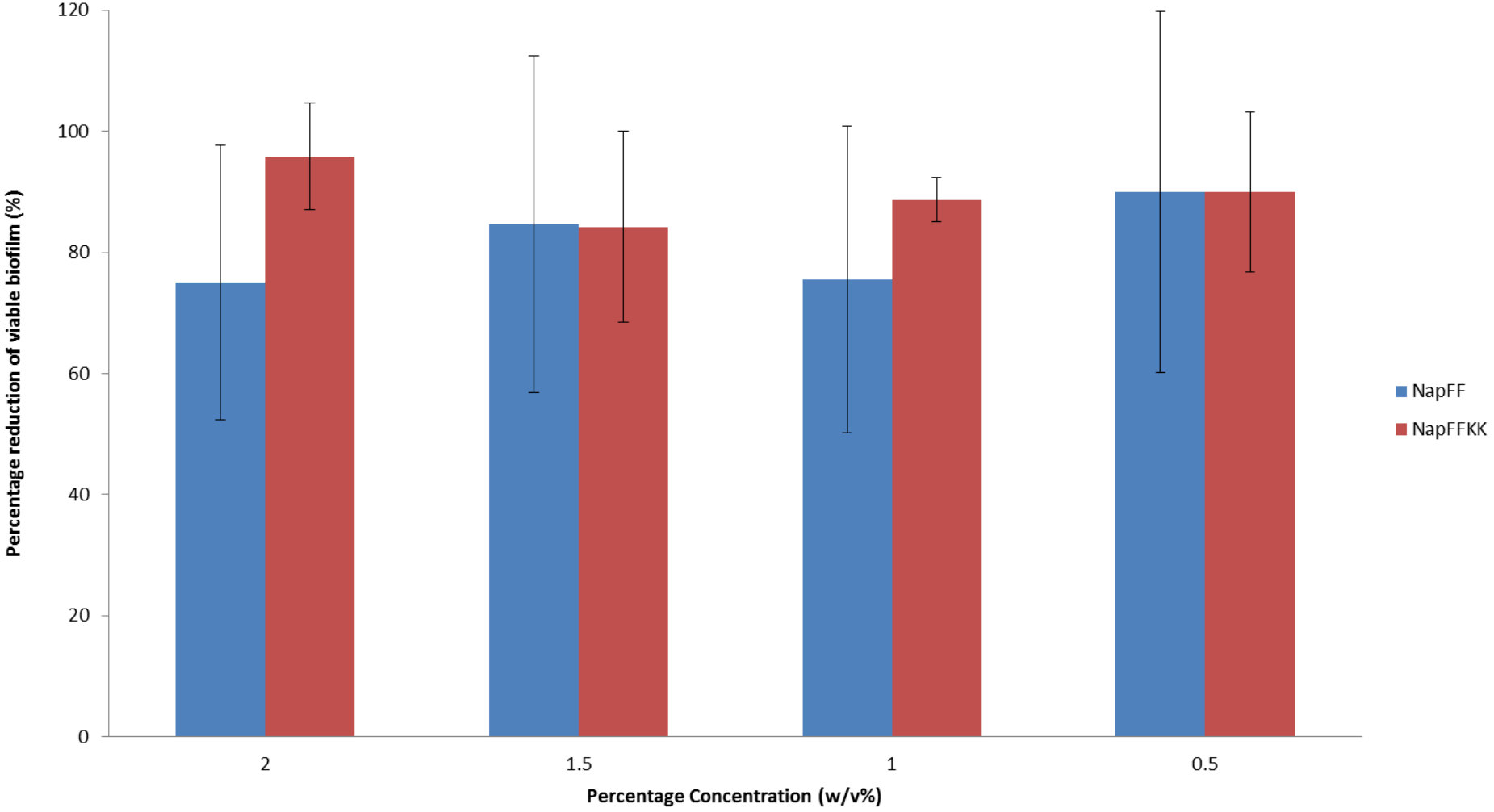
Percentage reduction of mature 24 hour *Pseudomonas aeruginosa* PA01 biofilm after 24 hour incubation with NapFF and NapFFKK self-assembled hydrogels utilising an alamarBlue assay. Results are displayed as a mean of 8 replicates.



Percentage reduction of mature 24 hour *Staphylococcus aureus* ATCC 6538 biofilm after 24 hour incubation with NapFF and NapFFKK self-assembled hydrogels utilising an alamarBlue assay. Results are displayed as a mean of 8 replicates.



Percentage reduction of mature 24 hour *Escherichia coli* ATCC 11303 biofilm after 24 hour incubation with NapFF and NapFFKK self-assembled hydrogels utilising an alamarBlue assay. Results are displayed as a mean of 8 replicates.



Future work

- PhD Student begin 30th September
- Test gels for:
  - Rheological properties
  - Structure (TEMs)
  - Structure activity relationship
  - Stability (Proteases/peptidases)
  - Bacterial counts (Modify MBEC assay)
  - *In vivo* analysis
- Development of a peptide hydrogel responsive to infection/biofilm formation

Acknowledgements

- Xu Group School of Chemistry Brandeis University
- Dr Brendan Gilmore and Prof. Sean Gorman, School of Pharmacy Queen's University Belfast